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Effect of simultaneous blockade of AT1 and AT2 receptors on the NFκB pathway and renal inflammatory response

Vanesa Esteban, Mónica Ruperez, Juan Rodriguez Vita, Elsa Sanchez López, Sergio Mezzano, Juan José Plaza, Jesús Egido, and Marta Ruiz-Ortega

Laboratory of Vascular and Renal Pathology, Fundación Jiménez Díaz, Universidad Autónoma, Madrid, Spain; and Universidad Austral, Valdivia, Chile

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Background. Angiotensin II (Ang II) is a cytokine that participates in the inflammatory response. The nuclear factor kappa B (NFκB) is involved in the regulation of many immune and inflammatory factors. Different works have shown that both angiotensin II receptor type 1 (AT_1) and type 2 (AT_2) receptors are involved in the NFκB pathway; however, some aspects remain mysterious. AT_1 antagonists increased plasma Ang II levels that could bind to AT_2, so understanding the clinical importance of AT_2 stimulation or inhibition is an interesting unresolved point.

Methods. Experiments were done in wild-type (WT) and AT_{1a} receptor knockout mice that received subcutaneous Ang II infusions (1000 ng/kg/min) for 3 days. Specific blockers of AT_1 (losartan 10 mg/kg/day) and AT_2 (PD123319 30 mg/kg/day) receptors were administered 1 day before and during Ang II infusion. NFκB activity was examined by electrophoretic mobility assay and inflammatory (monocyte/macrophage) cell infiltration by immunohistochemistry

Results. In WT mice, Ang II infusion caused renal NFκB activation that was partially diminished by either AT_1 or AT_2 antagonists. In AT_1 knockout mice, Ang II also activated renal NFκB, which was only blocked by the AT_2 antagonist. Both Ang II–infused WT and AT_1 knockout mice showed inflammatory infiltration in tubulointerstitial areas that were suppressed by the AT_2, but not AT_1, antagonist. Combined therapy of both AT_1 and AT_2 antagonists blocked renal NFκB activation and inflammatory cell infiltration, both in WT and in AT_1 knockout mice.

Conclusion. Ang II, via AT_1 and AT_2 stimulation, leads to NFκB activation that was only blocked by combined therapy with both antagonists. The participation of AT_2 receptors in the recruitment of inflammatory cells underscores the need of future studies that evaluate the clinical usefulness of this strategy.

Angiotensin II (Ang II) is a cytokine involved in the regulation of cell growth and fibrosis [1]. Several works have demonstrated that Ang II induces many proinflammatory events, regulates immune cell responses, and is involved in the pathogenesis of immune-mediated renal diseases [2, 3–5].

Two distinct subclasses of Ang II receptors, AT_1 and AT_2, have been described [6, 7]. The well-known Ang II actions (vasoconstriction, proliferation, and fibrosis) are mediated by the activation of various signal-transduction pathways via AT_1. The biological functions and molecular mechanisms of AT_2 remain mysterious. AT_2 signaling activates phosphatases and production of ceramides, arachinodate, and kinin/nitric oxide/cGMP, and regulates apoptosis/inhibition of cell growth, vasodilatation, and pressure natriuresis. This receptor is re-expressed in pathologic situations involving tissue remodeling or inflammation, including renal damage [6–8].

The nuclear factor kappa B (NFκB) is important in the regulation of the inflammatory response because it controls many proinflammatory genes, including cytokines, adhesion molecules, and chemokines [9]. Both in vivo and in vitro Ang II activate NFκB and up-regulate NFκB-related genes [2]. Recent works have demonstrated that both AT_1 and AT_2 receptors activate the NFκB pathway [10–14]; however, important questions remain unresolved [15, 16]. One such question is the biological significance of AT_2/NFκB activation. Some data suggest that AT_2 could be involved in the inflammatory cell recruitment [11, 17] and in the regulation of NFκB activity in the kidney [11, 13, 14]. When AT_1 is blocked, circulating Ang II levels are increased, free Ang II could bind to AT_2, and exert some yet undefined functions with potential clinical importance. The present studies were done to further investigate this issue, evaluating the role of Ang II receptors in renal inflammation and the NFκB pathway. For this purpose we used AT_{1a} receptor knockout mice and wild-type (WT) mice that were given systemic Ang II infusions, and the administration of selective nonpeptidic antagonists for AT_1 and AT_2 receptors, investigating what happened if both AT_1 and AT_2 receptors were blocked.

METHODS

Experimental design

Animals used were male WT (C57BL/6) and AT_{1a} receptor knockout mice, with the same genetic back-

ground. The AT_1 knockout was generated by a homologous recombination method (from Dr. Sugaya, Tanabe Seiyaku Corp., Osaka, Japan), and has been previously used [4]. Ang II was given by subcutaneous osmotic minipumps (Alza Corp., Cupertino, CA, USA), at a dose of 1000 ng/kg/min. Control (saline-infused) animals of the same age were also studied. Animals were sacrificed at days 3 ($N = 8$ in each group). Pharmacologic blockade of Ang II receptors was done with the AT_1 antagonist losartan (10 mg/kg/day; drinking water) or the AT_2 antagonist PD123319 (30 mg/kg/day, subcutaneous osmotic minipumps), or with a combination of both antagonists, starting 24 hours before Ang II infusion and maintained during the experimental period. The administered doses are known to effectively block the corresponding receptor [11]. Losartan was kindly provided by MSD (Madrid, Spain), and PD123319 was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Morphology and immunohistochemistry

Paraffin-embedded sections were treated for each technique as described before [11]. Morphology was evaluated by Masson's staining and light microscopy. Inflammatory cell infiltration was characterized by monoclonal antibodies against F4/80 antigen (Serotec, Raleigh, NC, USA), present in murine monocytes/macrophages. Briefly, sections were rehydrated, endogenous peroxidase and unspecific binding were blocked, incubated with primary antibodies, and revealed by standard methods. In each experiment, negative control groups (omitting the primary antibody, or with an unrelated antibody) were included. Immunohistochemical quantification for inflammatory cell infiltration was evaluated by image analysis using a KZ 300 Imaging System 3.0 (Zeiss, München-Hallbergmoos, Germany). The percentage of the stained area was calculated as the ratio of suitable binary threshold image and the total field area. For each sample, the mean staining area was obtained by analysis of 10 different fields (×20), excluding glomeruli and vessels. The staining score is expressed as percent positive area in the total area under examination.

Determination of renal NFκB activity

NFκB activity was evaluated by binding protein extracts from renal cortex with an oligoconsensus NFκB and analyzed by electrophoretic mobility shift assay (EMSA) [11].

Statistical analysis

Results are expressed as mean ± SEM. Significant differences were evaluated with non-parametric Kruskal-Wallis analysis of variance (ANOVA) tests using a commercial statistical program (GraphPAD Instat, San Diego, CA, USA). Differences were considered significant when the two-tailed P values was <0.05.

RESULTS

Angiotensin II infusion activates renal NFκB via AT_1 and AT_2 receptors

Systemic infusion of Ang II for 3 days increased NFκB DNA-binding activity in the kidney of WT mice (2.9-fold vs. control; $P < 0.05$, $N = 6$, EMSA) (Fig. 1). The AT_1 antagonist diminished around 66% renal NFκB activation, demonstrating that AT_1 is involved in the NFκB pathway. In Ang II–infused AT_1 knockout mice renal NFκB activity was increased but lower than in WT (2-fold, $N = 6$) (Fig. 1). The fact that AT_1 knockout mice still present activation of NFκB suggests the existence of an AT_1-independent NFκB activation. The contribution of AT_2 receptors to the stimulation of NFκB was further studied with its antagonist. PD123319 diminished renal NFκB activity, both in WT (around 66% inhibition vs. Ang II–infused mice) and AT_1 knockout Ang II–infused mice. These data demonstrate activation of the AT_2/NFκB pathway. When both AT_1 and AT_2 receptors were blocked, renal NFκB activity was reduced to control levels ($P < 0.05$ vs. Ang II; P = NS vs. control, $N = 3$).

AT_2 antagonist diminished inflammatory cell infiltration

The inflammatory cell infiltration was examined by immunohistochemistry with a specific anti-F4/80 antibody that recognizes murine monocytes/macrophages (Fig. 2). In control animals, only a few cells with positive anti-F4/80 immunostaining were observed. After 3 days of Ang II infusion, kidneys of WT and of AT_1 knockout mice presented an important increase in the mean number of monocytes/macrophages in the interstitium, distributed in a focal manner. Interestingly, there were no monocytes in the glomerular area, showing a clear difference with the Ang II infusion model in rats [11].

The effect of Ang II receptors blockade was evaluated. Interestingly, in WT only, the AT_2 antagonist, but not the AT_1, diminished renal inflammatory cell infiltration. Similar effects were also observed in AT_1 knockout, since the numbers of monocytes/macrophages were decreased to control levels by the AT_2 antagonist PD123319 (Fig. 2). In animals with combined therapy of PD123319 and losartan the number of monocytes was similar to control.

DISCUSSION

One of the molecular mechanisms elicited by Ang II and that could play a key role in the inflammatory response is the activation of the NFκB pathway [2]. In this work, we have demonstrated that in vivo Ang II activates NFκB via both AT_1 and AT_2 receptors, as shown by the results of inhibition with the corresponding specific antagonists. These data confirm our previous studies in the rat model of Ang II infusion [11]. In vascular smooth mus-

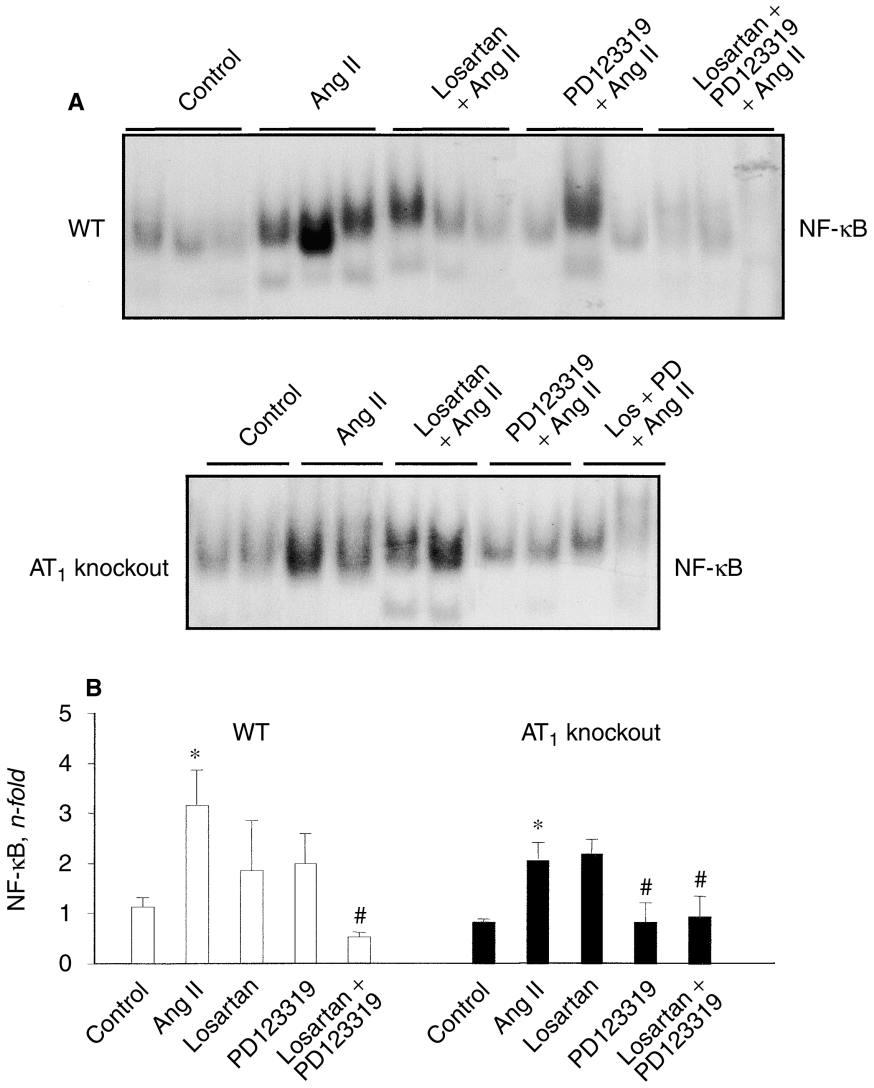

Fig. 1. Angiotensin II (Ang II) in vivo activates nuclear factor kappa B (NFκB) via AT_1 and AT_2 receptors. Wild-type (WT) and AT_{1a} receptor-knockout mice were infused with Ang II (1000 ng/kg/min) for 3 days and received the specific AT_1 antagonist losartan (10 mg/kg/day) or AT_2 antagonist PD123319 (30 mg/kg/day) alone or in combination. (*A*) Representative electrophoretic mobility shift assay (EMSA) experiment that shows 2 or 3 different animals of each group. Specificity of the reaction was demonstrated with a 100-fold excess of unlabeled NFκB oligonucleotide (not shown). (*B*) Densitometric analysis of the results (mean ± SEM of 4 to 6 animals in each group) expressed as arbitrary units. $*P < 0.05$ vs. control; $\#P < 0.05$ vs. Ang II infusion.

cle cells, glomerular mesangial cells, and endothelial cells, NFκB activation occurs both via AT_1 and AT_2 [10, 11, 14], while in tubuloepithelial cells it only occurs through AT_1 [11]. Studies using cells from AT_1 knockout mice have demonstrated that Ang II may activate NFκB independently of AT_1, as found in the present studies in the kidney of Ang II–infused AT_1 knockout mice. The existence of the AT_2/NFκB pathway has also been demonstrated with the use of specific AT_2 agonists, and more recently by Wolf et al [14] in studies in COS7 cells transfected with AT_2 receptors and PC12 cells, which only express AT_2. In those studies, the activation of the NFκB pathway was demonstrated by DNA binding and by transient transfection experiments with a reporter plasmid containing NFκB promoter binding sites.

The molecular mechanisms and cellular responses of Ang II receptors are not completely defined. AT_1 regulates vasoconstriction, cell proliferation, and production of cytokines and extracellular matrix proteins [1, 6, 7]. In models of renal injury, AT_1 antagonists decrease proteinuria, matrix accumulation, and the production of growth factors [1, 18]. The information provided by AT_2 blockers and AT_2 knockout mice supports an important role for the AT_2 in the physiopathology of the kidney [6, 7, 19, 20]. Some Ang II responses could be mediated by both AT_1 and AT_2 receptors, among them nitric oxide release, collagen synthesis, α-2-adrenoreceptor activity [21–23], and, as discussed here, NFκB activation.

The AT_2/NFκB pathway is likely involved in the regulation of the recruitment of inflammatory cells in the kidney. In this work, we have demonstrated that in Ang II–infused mice, only AT_2 blockade, but not AT_1, reduced the presence of monocytes in interstitial areas. Moreover, inflammatory cells were also observed in kidneys of Ang II–infused AT_1 knockout mice, which were also only diminished by AT_2 antagonist. In previous studies

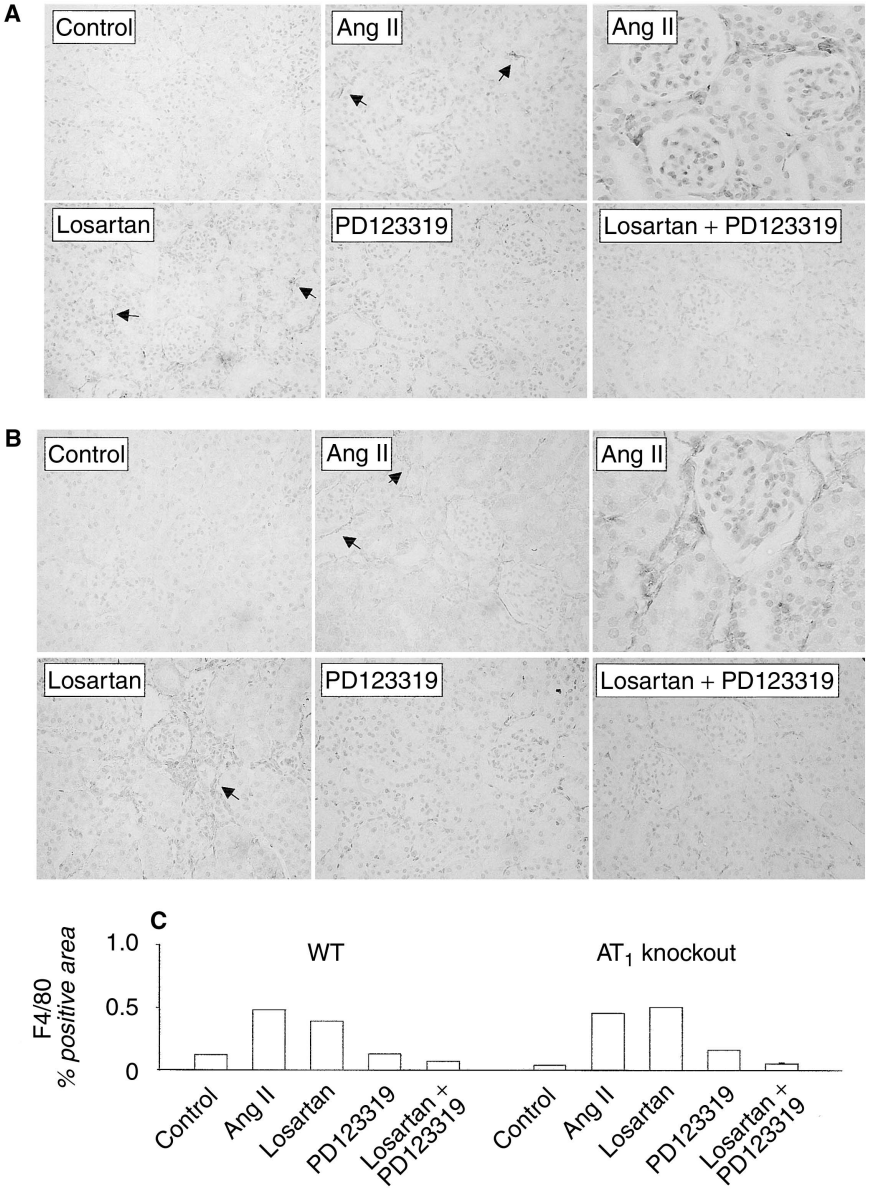

Fig. 2. Inflammatory cell infiltration in the kidney of angiotensin II (Ang II)–infused mice. The presence of monocytes/macrophages was determined by immunohistochemistry with an anti-F4/80 antibody. Figures show a representative animal of each group of wild type (*A*) and AT_{1a} receptor-knockout (*B*) mice (magnification ×100). Computer image analysis of monocyte/macrophage score in the kidney sections is demonstrated in (*C*) that shows results expressed as mean percent F4/80 positive area of 4 to 6 animals for each group.

in Ang II–infused rats, we and others have observed glomerular and interstitial inflammation that was only abolished by the AT_2 antagonists [11, 17]. This anti-inflammatory effect of AT_2 blockade may be mediated by the down-regulation of the expression of chemokines in glomerular and tubulointerstital areas [11, 17, 24]. For example, in glomerular endothelial cells Ang II increases regulated upon activation, normal T cell expressed and secreted (RANTES) expression via AT_2 receptors [17].

Other works also support the hypothesis that Ang II participates in the recruitment of inflammatory cells into the kidney via AT_2. Participation of AT_2-mediated responses may explain differences found in the inflammatory response between AT_1 antagonists and angiotensin-converting enzyme (ACE) inhibitors in some renal diseases. In the model of unilateral ureteral obstruction in rats, ACE inhibitors, but not AT_1 antagonists, diminished monocyte/macrophage infiltration [25]; in anti-thymocyte serum-induced nephritis, the AT_1 blockers only reduced inflammatory infiltration around 50% [26]. Similarly, in vascular diseases, some data also suggest that AT_2 participate in the inflammatory response. Pressure overload–induced cardiac hypertrophy was completely suppressed in AT_2 knockout mice [27], and studies on vascular lesions in AT_2 knockout mice have demonstrated that AT_2 mediates the effect of inflammation on vascular smooth muscle cell proliferation [28]. Furthermore, inflammatory mediators, including interleukin-1β (IL-1β), insulin, interferon regulatory factors, and Ang II up-regulate AT_2 expression [6, 29]. In several models of renal and

vascular damage characterized by an inflammatory response, AT_2 over-expression has been described [2, 6, 8]. These data further strengthen the possibility that AT_2 receptors are involved in the development of inflammatory process in renal and vascular diseases.

The Ang II receptor subtype involved in the immune and inflammatory response is not fully defined. Some experimental studies have shown that AT_1 antagonists diminished several proinflammatory parameters, including the cytokine interleukin-6 and adhesion molecules [2]. Ang II could activate leukocytes, leading to adhesion of these cells to the vascular endothelium and their migration into the vessel wall. Piqueras et al [30] have demonstrated that AT_1 and AT_2 receptor inhibition attenuated Ang II–stimulated leukocyte migration into rat mesenteric venules, showing that only combination of both receptor blockers returned all parameters to basal levels. We have also observed that only using combined treatment with AT_1 and AT_2 antagonists completely blocks renal inflammatory infiltration and NFκB activation in Ang II–infused mice. These data shows that the blockade of both receptors is necessary to fully stop the inflammatory response. In addition, Ang II can be degraded to other metabolites that can cause some pathologic effects, although there is little evidence of these mechanisms in vivo [31]. In this sense, we have also explored the possibility that other angiotensin-related peptides may possess proinflammatory properties. Treatment of cultured renal and mononuclear cells with the NH_2-terminal Ang II metabolite Ang III caused activation of NFκB and activator protein-1 (AP-1) and increased monocyte chemoattractant protein-1 (MCP-1) [32]. The Ang III–induced NFκB activation was mainly via AT_2 [13]. When AT_1 is blocked Ang II levels are increased and Ang peptides (Ang III, Ang IV, and Ang 1-7) can be formed [31]. These peptides, through binding to their specific receptors, might be involved in the effects of AT_1 blockade. In this sense, Ang III through AT_2 and the NFκB pathway could be involved in the inflammatory response, Ang(1–7) (maybe through a specific receptor) may cause vasodilatation, and Ang IV, via the AT_4 regulate plasminogen activator inhibitor-1 (PAI-1) expression [31], could participate in the pathogenesis of vascular damage.

The clinical importance of the finding that AT_2 blockade diminishes inflammatory cell infiltration and NFκB activation needs to be addressed in human studies. In untreated patients with hypertension, the ACE inhibitor enalapril decreased plasma levels of adhesion molecules and MCP-1, while losartan did not significantly change them [33]. Recent clinical trials in cardiovascular and renal diseases have shown that the beneficial effect of AT_1 antagonists and ACE inhibitors is not entirely explained by lowering blood pressure, but are also due to the inhibition of cellular Ang II actions [34–39]. Several experimental studies in animals and preliminary clinical studies indicate that the combination of both drugs may be more beneficial than either drug alone in hypertension, cardiac insufficiency, post-myocardial infarction, and type II diabetes. Large clinical trials are necessary to further document the putative advantages of such a combined therapy.

Several authors have defended the yin yang hypothesis, suggesting a counterbalance effect of AT_2 in the presence of AT_1 blockers. They suggest that AT_2 receptor activation leads principally to opposite effects to that observed when Ang II binds to AT_1 receptors, including vasodilatation and antiproliferative/apoptotic responses [6]. However, experimental studies with Ang receptor blockers and knockout mice have provided data that contradicts this postulate and the beneficial effects of two large clinical trials in heart failure can be explained without ascribing a major protective role to the unopposed activity of the AT_2 receptor in the failing myocardium [40]. Similar evidence was found in experimental models of renal damage (Ang II infusion and unilateral ureteral obstruction) that have demonstrated that AT_1 antagonists only partially diminished NFκB activation [11, 41]. Moreover, only when both AT_1 and AT_2 receptors are blocked, tissue NFκB activity is reduced to control levels, showing that both receptors may be involved in this molecular pathway. These data show that the beneficial effects observed with AT_1 antagonists cannot be attributed to stimulation of the AT2/NFκB pathway, destroying the yin yang–counterbalance idea, at least, in relation to NFκB/inflammation in the kidney.

CONCLUSION

Further experimental studies are necessary to define the role of Ang receptors in inflammatory and immune processes. In the progression from the bench to bedside, the ongoing clinical trials could afford important information of the potential beneficial effects of the blockade of AT_1 or AT_2 in human renal diseases.

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Reprint requests to Marta Ruiz-Ortega, Ph.D., Laboratory of Renal and Vascular Pathology, Fundación Jiménez Díaz, Avenida Reyes Católicos, 2 28040, Madrid, Spain.
E-mail: mruizo@fjd.es

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